JOURNAL OF INVERTEBRATE PATHOLOGY 61, 81–84 (1993)

Entomopathogenic Isolates of *Metarhizium anisopliae*, *Beauveria bassiana*, and *Aspergillus flavus* Produce Multiple Extracellular Chitinase Isozymes

RAYMOND J. ST. LEGER,[1] RICHARD C. STAPLES, AND DONALD W. ROBERTS

Boyce Thompson Institute for Plant Research, Tower Road, Cornell University, Ithaca, New York 14853-1801

Received February 25, 1992; accepted June 23, 1992

Multiple extracellular chitinase isozymes were detected in culture filtrates from *Metarhizium anisopliae, Beauveria bassiana,* and *Aspergillus flavus* following electrophoresis on sodium dodecyl sulfate–polyacrylamide gels. Following renaturation, lytic zones of glycol chitin included in the gel were revealed by UV illumination after staining with Calcofluor White M2R. The method was applied to a study on the mode of regulation of chitinolytic enzymes. High chitinase activities where present only in chitin-containing media. Addition of alanine, a readily utilized nutrient source, repressed chitinase production in *M. anisopliae* indicating that each isozyme was similarly regulated by products of chitin degradation by an inducer–repressor mechanism. However, a chitinase-deficient mutant of *M. anisopliae* demonstrated reduced production of all but one of the chitinase isozymes. The remaining activity (48 kDa) is evidently under separate control from the other isozymes which presumably are jointly controlled from a single regulatory gene. The implications of entomopathogens producing multiple forms of chitinase are discussed in terms of pathogenicity and future research prospects. © 1993 Academic Press, Inc.

KEY WORDS: ***Metarhizium; Beauveria; Aspergillus;*** **entomopathogens; multiple chitinases; regulation; analysis of mutants.**

INTRODUCTION

Both proteases and chitinase activities have been implicated as pathogenicity determinants involved in host invasion by entomopathogenic fungi (Charnley and St. Leger, 1991). The coincidental appearance of pathogen protease with cuticle degradation in infected insects suggests a more likely role for this enzyme (St. Leger *et al.*, 1988; Goettel *et al.*, 1989a). Attempts to relate the pathogenicity of various isolates with chitinase production *in vitro* (Yanagita, 1980; Bajan *et al.*, 1979) have given conflicting results because of the diversity of the genetic background of the isolates. Fungal chitinase mutants have previously been isolated following conventional mutagenesis, but with only limited success, partly because of insufficient awareness of the complexity of chitinase types and production (Jackson *et al.*, 1985; El-Sayed *et al.*, 1989). However, this is an appropriate time to undertake such studies because of the improved understanding of the enzymes and their regulation (St. Leger *et al.*, 1986, 1991; Vasseur *et al.*, 1990), and the development of techniques for detecting and characterizing the various chitinase isozymes produced by putative mutants (Trudel and Asselin, 1989).

Previously, we reported that *Metarhizium anisopliae* produces two distinct activities responsible for catalyzing hydrolysis of chitin and chitobiose (St. Leger *et al.*, 1991) and we reported on the regulation of these enzymes by chitin degradation products (St. Leger *et al.*, 1986). The comparatively few critical investigations conducted on regulation of production of fungal chitinases suggest that similar systems are also operating in saprophytes (Vasseur *et al.*, 1990; Ulhoa and Peberdy, 1991), but the complexity of chitinase types has not been investigated. In the present study, we report that *M. anisopliae, Beauveria bassiana,* and *Aspergillus flavus* produce multiple chitinase isozymes which are regulated by a chitin inducer–repressor system. We also demonstrated the use of isozyme analysis in the selection and characterization of chitinase-deficient mutants.

METHODS

Isolates of *M. anisopliae,* 23 (ex. *Conoderus vespertinus* Elateridae: Coleopt.) and 324 (ex. *Austracris guttulosa,* Acrididae: Orthopt.), *A. flavus* 1003 (ex. *Bombyx mori,* Bombycidae: Lepidopt.), and *B. bassiana* 252 (ex. *Leptinotarsa decemlineata,* Chrysomelidae: Coleopt.) were obtained from the USDA-ARS collection of entomopathogenic fungal cultures (USDA, Ithaca, NY). Strain 911 was obtained by ethane methane sulfonate mutagenesis of *M. anisopliae* 23 and demon-

[1] To whom reprint requests should be addressed.

0022-2011/93 $4.00
Copyright © 1993 by Academic Press, Inc.
All rights of reproduction in any form reserved.

strates diminished levels of chitinase (Al-Aidroos and Roberts, 1976) as confirmed in this study (Table 1).

Transfer experiments. Standardized mycelial inocula (5 g wet wt) from 48-hr Sabouraud dextrose broth cultures (St. Leger *et al.*, 1991) were incubated for up to 72 hr in 50 ml of sterile (120°C, 15 min) basal media (0.1% KH_2PO_4, 0.05% $MgSO_4$, pH 6) by itself (MM) or supplemented with chitin or cellulose at 1% (w/v). For some experiments, cultures were supplemented with alanine (1% w/v). Cultures were buffered at pH 6 with (2[*N*-morpholino]ethanesulfonic acid) (St. Leger *et al.*, 1986). For some experiments, cultures were supplemented with turkey egg white inhibitor (100 μg ml^{-1}), a specific inhibitor of Pr1 chymoelastase activity (St. Leger *et al.*, 1988).

Assays of cultures. Samples were taken from each flask at intervals, filtered through Whatman No. 1 filter paper, concentrated 10-fold by dialysis against polyethylene glycol (MW > 15,000), and centrifuged (1800*g*, 15 min, 4°C). Chitinase activity (vs colloidal chitin) in culture filtrates was assayed by measuring the release of reducing sugars (St. Leger *et al.*, 1986). Chymoelastase activity (Pr1) was assayed against succinyl-(alanyl)$_2$ prolyl phenylalanine *p*-nitroanilide (St. Leger *et al.*, 1988).

Preparation of chitin substrates. Colloidal chitin was prepared from crystalline chitin as described (St. Leger *et al.*, 1986). Glycol chitin, a soluble modified form of chitin, was obtained by acetylation of glycol chitosan by the method of Molano *et al.* (1979) as modified by Trudel and Asselin (1989). The glycol chitin was stored in a 1% (w/v) stock solution containing 0.02% (w/v) sodium azide.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Samples were boiled for 5 min in the loading buffer of Laemmli (1970) except β-mercaptoethanol was omitted (i.e., 15% (w/v) sucrose, 0.01% (w/v) bromophenol blue, and 2.5% (w/v) SDS in 125 mM Tris–HCl (pH 6.7)). SDS–PAGE was performed in 12.5% (w/v) polyacrylamide gels as described previously (St. Leger *et al.*, 1989) except for the inclusion of 0.01% (w/v) glycol chitin in the running gel.

Following electrophoresis, chitinase activities were detected by a modification of the method of Trudel and Asselin (1989). Gels were gently shaken for 20 hr at 30°C in 100 mM sodium acetate buffer (pH 5.3) containing 1% (v/v) Triton X-100. Gels were then stained with 100 ml of freshly prepared 0.01% (w/v) Calcofluor White M2R in 500 mM Tris–HCl (pH 8.9). After 10 min, the brightener solution was removed and the gels were shaken for 1 hr in frequent changes of distilled water. Lytic zones representing digested chitin were visualized by placing the gels on a Fotodyne UV box and photographed (exposure time: 10 sec) with Polaroid Type 55 film, UV-haze, and a Type 85B filter. The number of bands was counted by visual examination.

Chemicals. Turkey egg white protease inhibitor, technical grade chitin, fibrous cellulose (medium) glycolchitosan, and *Streptomyces griseus* chitinase were from Sigma. All other chemicals used were of analytical grade and were supplied by Sigma or BDH.

RESULTS

Mycelia of *M. anisopliae, B. bassiana,* and *A. flavus* produced extracellular chitinase activities which appeared between 30 and 40 hr of being transferred to a media containing chitin as sole carbon and nitrogen source. Cultures were harvested early (40 hr) for isozyme analysis, as activities are more likely to represent secreted enzymes without any additional enzymes which may be produced during autolysis (≥72 hr, determined microscopically). The presence of an insoluble chitin substrate precluded accurate measurements of growth. This problem was alleviated by using standardized mycelial inocula to ensure a large active biomass.

The chitinase activities of *M. anisopliae* (23), *B. bassiana,* and *A. flavus* detected in 40-hr cultures (Table 1) are represented by 10, 18, and 6 isozymes, respectively (Fig. 1). At least 20 apparent chitinase isozymes are

TABLE 1

Effect of Different Carbon Sources on Extracellular Chitinase Production in 40-hr Cultures of *M. anisopliae, B. bassiana,* and *A. flavus*

	M. anisopliae				
Carbon source[a]	23	23 > 911	324	*B. bassiana*	*A. flavus*
None (MM)	0	0	0.09 ± 0.02	0	0
1% cellulose	0	0	0.11 ± 0.03	0	0
1% chitin	0.53 ± 0.07[b]	0.06 ± 0	2.43 ± 0.26	0.67 ± 0.07	0.40 ± 0.08
1% chitin plus 1% alanine	0.04 ± 0	0	0.34 ± 0.02	0.04 ± 0.01	0.07 ± 0.02

[a] All cultures were inoculated with standardized mycelial inocula (5 g wet wt) (Methods).

[b] Chitinase activities expressed as units (μmol GlcNac ml^{-1} hr). Each result is the mean of three replicates ± SD. The experiment was repeated once (data not shown) with comparable results.

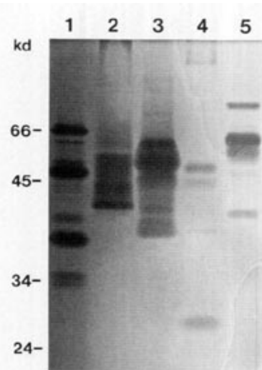

FIG. 1. Chitinase activity after SDS–PAGE in a gel containing 0.01% (w/v) glycol chitin as substrate. Commercial *Streptomyces griseus* chitinase (lane 1) was used as a control for known chitinase activity. Filtrates from chitin-grown cultures of *B. bassiana* (lane 2), *M. anisopliae* 324 (lane 3), *A. flavus* (lane 4), and *M. anisopliae* 23 (lane 5) were separated in 12.5% acrylamide gel and enzymes renatured in buffered Triton X-100; 50 μg of protein was applied to each well except for lane 1 (3 μg). The results are representative of three similar experiments.

produced by *M. anisopliae* 324 when grown on 1% chitin/basal salts media (Fig. 1). Synthesis of all isozymes by 324 was reduced when chitin media was supplemented with alanine (Fig. 2), a readily utilized nutrient source resulting in an 86% drop in total extracellular chitinase (Table 1). This result confirms that catabolite repression overrides the inducing effect of chitin. Chitinase was produced in trace amounts in unsupplemented basal salts or cellulose/basal salts (Table 1 and Fig. 2), confirming the specificity of induction by chitin components. Likewise, strain *M. anisopliae* (23), *B. bassiana,* and *A. flavus* produced chitinase in chitin media but not in cellulose or unsupplemented basal salts media (Table 1). The reduced chitinase activity produced by mutants could stem from the loss of one or more of the isozymes; alternatively, synthesis or release of all of the isozymes could be impaired. The role of individual isozymes or specific combinations in disease may be of greater importance than overall chitinase activity; thus chitinase isozyme profiles were determined for a chitinase-deficient mutant (911) previously investigated in pathogenicity studies (Al-Aidroos and Roberts, 1976). Mutant 911 apparently produced wild-type levels of a major chitinase isozyme (48 kDa) although all other isozymes were produced at very low levels (Fig. 2).

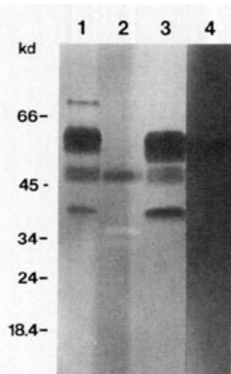

FIG. 2. SDS–PAGE (12.5% w/v, acrylamide) analysis of chitinases from wild-type *M. anisopliae* 23 (lane 1) and the chitinase-deficient mutant 911 (lane 2) and *M. anisopliae* 324 grown in 1% chitin media (lane 3) or 1% chitin media supplemented with 1% alanine (lane 4). No chitinase activity was detectable when *M. anisopliae* 23 or 324 were grown in 1% cellulose or unsupplemented basal salts media (lanes not shown); 50 μg of protein was applied to each well. The results are representative of three similar experiments.

To investigate the influence of Pr1 protease, isozyme production by *M. anisopliae* (23), *B. bassiana,* and *A. flavus* was examined in cultures supplemented with turkey egg white inhibitor at a level (100 μg ml^{-1}) which completely inhibited chymoelastase activity. No changes were observed in the profiles of isozyme production by the three fungi, indicating that chitinases are refractory to proteolysis by pathogen enzymes.

DISCUSSION

The results of this study suggest that fungi produce a previously unsuspected complexity of chitinase types coordinately induced by chitin components. Mutants deficient in chitinase activity could arise by impaired secretion or deficiency in a regulatory component such as loss of an intracellular inducer-generating enzyme. However, critical assessment of the role of isozymes will be through the use of structural gene mutants. In the case of an activity existing as multiple isozymes, these are likely to be obtainable only by *in vitro* mutagenesis, as a deficiency due to mutation in a structural gene will be difficult to detect phenotypically (i.e., by screening total activity) because of masking by numerous other isozymes which remain unaffected.

A chitinase-deficient mutant (911) showed reduced production of all but one of the chitinase isozymes. The remaining 48-kDa activity is evidently under separate control from the other isozymes which presumably are jointly controlled from a single mutated (in 911) regulatory gene. Analysis of isozyme patterns provides an essential way of establishing the nature of chitinase-deficient mutants once they are obtained. Each species that we studied produced a heterogenous collection of chitinases which may conceivably play an important role in their ability to adapt to different environments including that provided by the insect host. Most of the bands observed are probably the product of at least one structural gene. The multitude of isozymes is unlikely to be the result of post-translational modifications (e.g., glycosylation) given the wide range of molecular weights observed. Given the number of genes involved, the probability of simultaneously neutralizing all of these by mutagenesis is remote; thus secretory mutants or regulatory mutants are more likely. Mutant 911 secretes normal levels of Pr1 protease (unpub-

lished data) as well as the 48-kDa chitinase, indicating that it is unlikely to be a secretory mutant. The large number of chitinase isozymes makes these species unsuitable for the isolation of specific chitinase mutants until the establishment of site-directed mutagenesis with fungal chitinase genes. Obviously, it would still greatly facilitate the performance of such studies to perform a careful choice of pathogens, based partly on enzyme multiplicity. The ideal choice would be to work on a system containing just one or two gene copies. The recent developments in transformation technology and gene cloning in entomopathogenic fungi (Goettel *et al.*, 1989b; St. Leger *et al.*, 1992a,b) could then be applied to genetic manipulations of these chitinase genes.

REFERENCES

Al-Aidroos, K., and Roberts, D. W. 1976. Mutants of *Metarhizium anisopliae* with altered virulence toward mosquito larvae. *In* "Proceedings, 1st Intern. Colloq. Invertebr. Pathol., Queen's Univ., Kingston, Ontario", pp. 423–424.

Bajan, C., Kalalova, S., Kimitowa, K., Samsinakova, A., and Wojciechowska, M. 1979. The relationship between infectious activities of entomophagous fungi and their production of enzymes. *Bull. Acad. Pol. Sci. Cl. 2 Ser. Sci. Biol.* 27, 963–968.

Charnley, A. K., and St. Leger, R. J. 1991. The role of cuticle-degrading enzymes in fungal pathogenesis in insects. *In* "The Fungal Spore and Disease Initiation in Plants and Animals" (G. T. Cole and H. C. Hoch, Eds.). Plenum, New York, pp. 267–286.

El-Sayed, G. N., Coudron, T. A., and Ignoffo, C. M. 1989. Chitinolytic activity and virulence associated with native and mutant isolates of an entomopathogenic fungus *Nomuraea rileyi*. *J. Invertebr. Pathol.* 54, 394–403.

Goettel, M. S., St. Leger, R. J., Rizzo, N. W., Staples, R. C., and Roberts, D. W. 1989a. Ultrastructural localization of a cuticle-degrading protease produced by the entomopathogenic fungus *Metarhizium anisopliae* during penetration of host (*Manduca sexta*) cuticle. *J. Gen. Microbiol.* 135, 2233–2239.

Goettel, M. S., St. Leger, R. J., Bhairi, S., Jung, M. K., Oakley, B. R., and Staples, R. C. 1989b. Transformation of the entomopathogenic fungus *Metarhizium anisopliae* using the *Aspergillus nidulans* benA3 gene. *Curr. Genet.* 17, 129–132.

Jackson, C. W., Heale, J. B., and Hall, R. A. 1985. Traits associated with virulence to the aphid *Macrosophoniella sanborni* in eighteen isolates of *Verticillium lecanii*. *Ann. Appl. Biol.* 106, 39–48.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227, 680–685.

Molano, J., Polacheck, I., Duran, A., and Cabib, E. 1979. An endochitinase from wheat germ. *J. Biol. Chem.* 254, 4901–4907.

St. Leger, R. J., Cooper, R. M., and Charnley, A. K. 1986. Cuticle-degrading enzymes of entomopathogenic fungi: Regulation of production of chitinolytic enzymes. *J. Gen. Microbiol.* 132, 1509–1517.

St. Leger, R. J., Durrands, P. K., Charnley, A. K., and Cooper, R. M. 1988. Role of extracellular chymoelastase in the virulence of *Metarhizium anisopliae*. *J. Invertebr. Pathol.* 52, 285–293.

St. Leger, R. J., Butt, T. M., Staples, R. C., and Roberts, D. W. 1989. Synthesis of proteins including a cuticle-degrading protease during differentiation of the entomopathogenic fungus *Metarhizium anisopliae*. *Exp. Mycol.* 13, 253–262.

St. Leger, R. J., Staples, R. C., and Roberts, D. W. 1991. Changes in translatable mRNA species associated with nutrient deprivation and protease synthesis in *Metarhizium anisopliae*. *J. Gen. Microbiol.* 137, 807–815.

St. Leger, R. J., Frank, D. C., Roberts, D. W., and Staples, R. C. 1992a. Molecular cloning and regulatory analysis of the cuticle-degrading protease structural gene from the entomopathogenic fungus *Metarhizium anisopliae*. *Eur. J. Biochem.* 204, 991–1001.

St. Leger, R. J., Staples, R. C., and Roberts, D. W. 1992b. Cloning and regulatory analysis of starvation-stress gene, *ssgA*, encoding a hydrophobin-like protein from the entomopathogenic fungus, *Metarhizium anisopliae*. *Gene* 120, 119–124.

Trudel, J., and Asselin, A. 1989. Detection of chitinase activity after polyacrylamide gel electrophoresis. *Anal. Biochem.* 178, 362–366.

Ulhoa, C. J., and Peberdy, J. F. 1991. Regulation of chitinase synthesis in *Trichoderma harzianum*. *J. Gen. Microbiol.* 137, 2163–2169.

Vasseur, V., Arigoni, F., Andersen, H., Defago, G., Bompeix, G., and Seng, J. M. 1990. Isolation and characterization of *Aphanocladium album* chitinase-overproducing mutants. *J. Gen. Microbiol.* 136, 2561–2567.

Yanagita, T. 1980. The formaldehyde resistance of *Aspergillus* fungi attacking silkworm larvae. 4. The relationship between pathogenicity to silkworm larvae and chitinase activity of *Aspergillus flavus oryzae*. *J. Seric. Sci. Jpn.* 49, 440–445.